NeoBiota 88: 103–133 (2023)
doi: 10.3897/neobiota.88.98440
https://neobiota.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
NeoBiota
Advancing research on alien species and biological invasions

Defining the biosecurity risk posed by soil found on sea freight

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Academic editor: C. Daehler | Received 14 December 2022 | Accepted 19 September 2023 | Published 19 October 2023

Citation: McNeill MR, Phillips CB, Richards NK, Aalders LT, van Koten C, James TK, Young SD, Bell NL, Laugraud A (2023) Defining the biosecurity risk posed by soil found on sea freight. NeoBiota 88: 103–133. https://doi.org/10.3897/neobiota.88.98440

Abstract

Soil associated with sea freight (shipping containers, flat racks and used machinery) arriving at New Zealand seaports was sampled for bacteria, fungi, nematodes, macro-invertebrates and plant seeds. Pseudomonads were selectively isolated, as several significant plant pathogens fall within this bacterial group. The mean and median sample weight collected from sea freight was found to be 417.3 and 152.7 g, respectively, with most recovered soil (73%) collected from the underside of shipping containers and flat rack containers. Likewise, for used machinery, most recovered soil (75%) was found under the machinery. Flat rack containers had significantly higher soil contamination compared to shipping containers and used machinery, but generally the counts and incidence of taxa were significantly lower compared to these other freight types. Viable bacteria, fungi, nematodes, seeds and arthropods were associated with the soil, with both counts g^{-1} and prevalence in samples varying with taxa, freight type, and location on the freight. Various regulated biosecurity organisms were recovered from the samples, including *Aphelenchoides besseyi* (rice white tip nematode), and seeds from genera such as *Brachiaria*, *Cortaderia*, *Digitaria*, *Eragrostis* and *Sonchus*. There were also live arthropod taxa that were not recorded as being present in New Zealand. No known plant pathogenic pseudomonads were identified through sequencing of the 16S ribosomal RNA gene. Shipping containers were found to be an important introduction pathway for exotic species, and therefore require careful monitoring and management. Comparisons of the incidence and mean number of organisms associated with soil on sea freight compared to a previous study with soil on footwear, generally showed that incidence and counts of many taxa were lower on sea freight, indicating that biosecurity

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risk can vary with pathway. However, prioritising one soil pathway over another according to the risks they present, and differentially allocating resources is problematic because the relative risk is dynamic, dictated by factors such as new pests or diseases entering the respective pathways.

Keywords
Biological invasions, biosecurity, flat rack, invasive alien species, pathway risk factors, pest risk analysis, shipping containers

Introduction

Globalisation is playing a major role in the spread of invasive alien species (IAS) with profound consequences for environments, economies, and human welfare (Bright 1999; Mack et al. 2000; Early et al. 2016; Diagne et al. 2020). Failure to address biological invasions is expected to result in continued increases in the number of species establishing beyond their native ranges (Levine and D'Antonio 2003; Westphal et al. 2008; Hulme 2009), because establishment rates of IAS show no sign of saturation and those of some taxa may even be increasing (Seebens et al. 2018). International maritime shipping has long been recognised as a pathway for spreading terrestrial IAS including arthropods, vertebrates and plants (Stanaway et al. 2001; Shimono and Konuma 2008; Sardain et al. 2019; Lucardi et al. 2020; Dawson et al. 2022), as well as marine species through biofouling and ballast water (Bax et al. 2003; Briski et al. 2013; Ojaveer et al. 2018; Letschert et al. 2021).

While shipping containers (e.g., typically a 20' twenty-foot equivalent unit (TEU)), or 40' forty-foot equivalent unit (FEU), have increased the volume and efficiency of trade (Reiter 2010), they have also exacerbated the risk of spreading IAS (Catley 1980), including plant pathogens (Gadgil et al. 2000), soil dwelling organisms (Godfrey and Marshall 2002), arthropods (Gadgil et al. 2000; Stanaway et al. 2001; Ward et al. 2006; Fenn-Moltu et al. 2022), reptiles, and amphibians (Kraus 2007; Chapple et al. 2013). The risk posed by soil contaminants on sea freight (e.g., shipping containers, cars, used machinery) is recognised, but is not well understood because IAS in soil are often cryptic and their survival partly depends on environmental factors. Freight arriving at seaports can become contaminated with soil and plant material before shipping, during transport to seaports, while being loaded on ships, and through cross contamination between objects such as stacked containers (Hampson and Wood 1997; Jennings et al. 1997; Hughes et al. 2010). For instance, random sampling of 3681 shipping containers at New Zealand seaports between September 1997 and May 1998, found that 23.4% carried quarantinable contaminants such as soil, viable insects, and plant pathogens (Gadgil et al. 2000).

Environmental exposure can have significant effects on IAS abundance and survival in soil associated with sea containers. For example, a study examining the fate of soil deliberately placed on the roof, under or inside four land-based shipping containers, found that the viability of *Pseudomonas* spp., nematodes and seeds in the soil on

the roof declined over 12 months, with mortality higher for most biota when the soil was exposed to sunlight, moisture or desiccation than when protected (McNeill et al. 2017). Conversely, there were more bacteria and fungi in soil situated in exposed environments, possibly due to ongoing colonization of exposed soil by airborne propagules (McNeill et al. 2017). A study of the microbiota from soil sampled from footwear in passengers' luggage showed high incidences and counts g^{-1} of seeds, nematodes, live arthropods, bacteria and fungi, which were comparable to fresh garden soil, probably because they had been protected from potential mortality factors such as extreme temperatures, moisture, desiccation and ultraviolet light (McNeill et al. 2011).

The research reported here examined the biological risk posed by soil on the external surfaces of sea freight (shipping containers, flat rack containers, and used machinery) and compared the results to those from our previous studies on soil biota on contaminated footwear (McNeill et al. 2011) and effects of storage time and environmental exposure on survival of soil biota (McNeill et al. 2017). The outcomes facilitate assessment of relative risks between and within pathways, contributing to the development of recommendations for increasing the efficiency and efficacy of biosecurity interventions.

Methods

Soil sample collection and processing

Shipping containers, flat rack containers and used machinery (hereafter collectively referred to as 'sea freight') arriving at two New Zealand seaports, Auckland (36°50.766'S, 174°47.154'E) and Lyttelton (43°36.51'S, 172°43.626'E), were examined and sampled for soil contamination between March and November 2011 and May and June 2012. In New Zealand, biosecurity management of sea freight involves risk profiling prior to arrival. Most freight is free of contamination having been thoroughly cleaned prior to shipping but, where contamination is suspected, freight is visually inspected once it has been offloaded at the port. Therefore, potentially contaminated sea freight was identified prior to arrival through risk profiling by New Zealand's Ministry for Primary Industries (MPI), which is the country's National Plant Protection Organisation (NPPO). Additional contaminated sea freight was identified by port stevedores as it was being unloaded. As the aim of the study was to understand the inherent risk posed by soil contaminants, not incidence of risk, sampling was not random but guided by these processes. Individual shipping containers and flat rack containers were placed on a raised stand with a forklift and all six external sides were visually inspected for contaminants. The location of the contaminants varied but were collected from one or more of the following areas on each container: the roof, outer rail, underside (inner rails or bottom of container), forklift pockets or corner castings. (See Suppl. material 1: fig. S1 for location of collection sites on shipping containers). For flat rack containers, the freight being carried was also inspected. The sides, undersides and tops of used

machinery were inspected, and any contaminants were collected. Soil was collected using either a paint scraper or a plastic hearth shovel and brush, and initially scooped into a sealable plastic container (330 mm L × 330 mm W × 127 mm H) before being transferred to a labelled sealable plastic bag. Where contaminants were collected from more than one location on a container or used machinery, the samples were kept separate. Where practicable, all soil was collected with collection equipment sterilised between samples using 70% ethanol.

Where possible, details were recorded on country of origin of the freight based on the shipping manifest, departure date from the last port of call (which in some cases was different from shipping container country of origin), and arrival date at Auckland or Lyttelton Port. Contamination may not have occurred in the country where the freight originated, as containers may be loaded, unloaded and reloaded at multiple ports prior to arrival in New Zealand. However, as in Gadgil et al. (2000), the last port of loading was used as an indicator of the probable origin of the soil contamination.

Sampling at the ports was carried out in conjunction with biosecurity personnel from MPI. All samples were transferred under a quarantine permit and processing was conducted in PC2 (quarantine) laboratories and glasshouses. Consistent with New Zealand's biosecurity standards, all biological material was destroyed once culturing or rearing, and identification was completed.

In the laboratory, samples were weighed using a bench scale (0–5000 g), then carefully examined under a stereo microscope at a 10–40× magnification to assess the nature of the soil (e.g., rocky, sandy, loess) or coral, and for the presence of biological material (e.g., seeds or arthropods) and other artefacts (e.g., glass, plastic, synthetic fibres).

Macro-invertebrates

Insects, mites, spiders, arthropod body parts, and molluscs observed under the stereo microscope were removed and counted. When live specimens were found either at the port during sampling or under the stereo microscope, they were collected and killed in 98% ethanol for subsequent identification using the expertise of New Zealand taxonomists.

Culturable microorganisms

Depending on sample weight, a subsample of between 0.2 (from small volumes of soil) to 21.0 g (for samples over 500 g) was taken to estimate densities (colony forming units per g or CFU g^{-1}) of bacteria, fungi, and *Pseudomonas* spp. Each sample was diluted 10-fold using 0.1% peptone and sonicated for 3 minutes to facilitate mixing. Serial dilutions were plated onto three different media: 10% tryptic soy agar plates with 100 mg/L cycloheximide to determine total culturable bacteria counts g^{-1} (hereafter referred to as counts); water agar containing 100 mg/L streptomycin for total culturable fungi counts; and Oxoid *Pseudomonas* selective agar (PSA), selective media

for growth and isolation of *Pseudomonas* bacteria supplemented with Oxoid CFC (cetrimide 10 mg/L, fucidin 10 mg/L and cephalosporin 50 mg/L). For total bacteria and *Pseudomonas* spp. isolation, plates were incubated at 20 °C (0:24 h L: D) and colonies counted after seven days. For fungi, plates were incubated at 20–25 °C (0:24 h L: D) and colonies counted after 10 days. For counting, the lowest detection limit was 100 microbial colonies per gram of soil (100 CFU g^{-1}). *Pseudomonas* bacteria were chosen as the 'model' microorganism for isolation as the genus includes several important plant pathogens (Moore et al. 2006; Silby et al. 2011), with some classified as regulated organisms by MPI.

Molecular identification of *Pseudomonas* bacteria

Sequencing of the 16S ribosomal RNA (rRNA) gene was used to diagnose species from a subset of colonies growing on the *Pseudomonas* selective agar. Colony morphology was visually assessed using characteristics such as colour, shape, and texture, and only the most prevalent colony type, based on its appearance was selected for molecular identification. The dominant colony type for each soil sample was purified by streaking onto nutrient agar and incubated at 20 °C and 0:24 h L: D for three days. Where more than one colony type was prevalent in high numbers for the same soil sample, then up to three morphologically different colonies were selected for further purification onto nutrient agar. A 5 mL nutrient broth culture was inoculated using a single colony from each selected isolate and incubated overnight at 20 °C on a shaker set at 200 rpm. A half mL of each overnight culture was transferred into 0.5 mL of sterile 30% glycerol in a 1.7 mL microcentrifuge tube, sealed with Parafilm M (Bemis Company, Wisconsin USA) and sent to Macrogen Inc. (Seoul, South Korea) for partial 16S rRNA sequencing. Macrogen performed the genomic DNA extraction, amplified the 16S rRNA gene using universal primers 27F and 1492R, then sequenced an internal region of this amplicon using universal primers 518F and 800R.

The sequences were tidied using Geneious version Prime 2021.2 (Kearse et al. 2012) and compared to *Pseudomonas* reference sequences on the Genbank database (Clark et al. 2016) using a BLAST search (Altschul et al. 1997). Multiple alignments of the sea freight isolate sequences and reference species were generated using the program Muscle (Edgar 2004). Visualisation of the genetic diversity between the isolates and reference sequences was conducted in MEGA11 (Tamura et al. 2021). Genetic distance was calculated by the Jukes-Cantor model (Jukes and Cantor 1969) and visualised using a neighbour-joining tree (Saitou and Nei 1987). The robustness of the nodes was assessed with 1000 bootstrap replicates (Felsenstein 1985). Each isolate was assigned to one of three lineages (*P. aeruginosa*, *P. fluorescens* and *P. pertucinogena*) and 14 phylogroups previously described (Mulet et al. 2010; Peix et al. 2018; Passarelli-Araujo et al. 2022). More rigorous methods of diagnosing *Pseudomonas* species using concatenated 16S rRNA and house-keeping genes (Mulet et al. 2010) was beyond the scope of this study.

Nematodes

For each sample, extraction of nematodes followed the method of Bell and Watson (2001) using the Whitehead and Hemming tray method whereby each sample was placed on two layers of paper tissue (Kimwipes™, Kimberley-Clark Worldwide Inc), supported by two layers of plastic mesh, the top and bottom mesh, 1mm and 44mm square, respectively, which were placed within a shallow tray. The tissue was folded to form an envelope and tap water was added to just cover the envelope. Nematodes were collected from the soil for 72 hours then the liquid was poured into a 1000 mL glass beaker, left to settle for 3–4 hours, and gently reduced to ~75 mL by removing the supernatant. The 75 mL samples were transferred to 100 mL glass Schott bottles and allowed to settle for 3–4 hours before being reduced to a final volume of 20 mL. Nematodes including plant parasitic nematodes (PPN), were counted and identified morphologically using a light microscope at 50× magnification and sorted into feeding groups (e.g., bacterivores, fungivores, predators, omnivores and plant feeders), and where possible genera or family, based on the keys of Siddiqi (2000) for Tylenchida, and Bongers (1994) for other groups. One or two specimens from each feeding group were preserved in a 4% formaldehyde solution containing 1% glycerol and mounted onto slides to enable identification at a 400× magnification. The weight of the subsamples from which nematodes were extracted depended on overall sample size after a subsample had been removed for microbial assessment, and ranged from 0.9 g to 1895 g.

Molecular identification of nematodes

In addition to samples taken for morphological identification, one or two specimens from each nematode feeding group were also genetically identified. Specimens chosen for DNA sequencing were mostly from taxa within the Tylenchida order, which includes plant parasitic nematodes of biosecurity concern, but specimens from taxa within other feeding groups were also sequenced to confirm morphological identifications. DNA was extracted from single specimens using the prepGEM™ tissue kit (ZyGEM Corporation Ltd., New Zealand) according to the manufacturer's instructions. For all specimens, the primers SSU 18A and 18P (Blaxter et al. 1998), and 1165SR (Ross et al. 2010) were used to target a portion of 18S small subunit rDNA gene. For spiral nematodes, the D2A and D3B primers (Subbotin et al. 2007) were used to target the D2-D3 region of the large subunit rDNA. DNA was amplified in 25µl reactions using 1× buffer (Thermo Scientific Finnzymes), 0.2 mM dNTPs, 0.3 µM of each primer, 0.2 mg/mL BSA and 0.5 units of Phusion Hot Start II Hi-Fi DNA polymerase (Thermo Scientific Finnzymes). Thermo cycling included an initial denaturing at 98 °C for 2 minutes, then for 40 cycles, 98 °C for 10 seconds, 57 °C for 30 seconds, 72 °C for 60 seconds, with a final extension step of 72 °C for 5 minutes. The product was purified using the EZNA Cycle-Pure kit V-spin (Omega Bio-tek). Fragments were sequenced by Massey Genome Service (Massey University, New Zealand) and cleaned using the computer programme Sequencher 4.6 (Gene Codes Corporation, USA) and Geneious 10.0.9 (Kearse et al. 2012). Sequences were compared to nematode sequences on the Genbank database (Clark et al. 2016) using a BLAST search (Altschul et al. 1997).

Seeds

Seeds were recovered during the initial laboratory inspection of samples under a binocular microscope, counted, and forwarded to the New Zealand National Seed Laboratory for identification and viability testing. Most seeds were identified to at least genus. The plant type (e.g., grass, herb, tree) to which the seed belonged was classified using those in 'Flora of New Zealand' (Webb et al. 1988; Edgar and Connor 2000). Viability was assessed either by dissecting the seed to observe the cotyledon, or by squashing the seed to determine if it had a milky white consistency indicating a healthy endosperm.

Because visual searches are imperfect at detecting all seeds in soil samples (e.g., McNeill et al. 2011), soil used for nematode extraction (described above) was kept allowing viable seeds to germinate. Small (< 100 g) subsamples were spread in a 5 mm thick layer on a paper towel over moist potting mix. Larger subsamples were spread on a layer of moist paper towels in a small tray. The soil was kept moist under natural light in a PC2 glasshouse (15–35 °C) and checked 1–3 times per week for seedlings, which were counted and if necessary transplanted into sterile potting mix 1–2 days after emergence and allowed to grow further for identification. When plants flowered, voucher specimens were collected and transferred to the Allan Herbarium PC2 specimen reception (Landcare Research, Canterbury Agriculture & Science Centre, Lincoln, New Zealand). Identifications were made using web-based keys (e.g., http://www.efloras.org/index.aspx) and publications relevant to the origin of each subsample (e.g., Whistler 1995). For each subsample, seeds recovered during visual searches and seedlings observed in germination tests were counted and adjusted to seeds per gram of soil.

Molecular identification of seeds

For plants in which the morphological identification was uncertain, DNA was extracted from leaf tissue using a Qiagen DNeasy Plant Mini kit (Qiagen Ltd., New Zealand) following the manufacturer's instructions. DNA samples were PCR amplified and sequenced for the following chloroplast and ribosomal nuclear regions: two internal transcribed spacers (ITS1 and ITS2) that flank the 5.8 S nuclear ribosomal DNA region (White et al. 1990); chloroplast trnL-trnF spacer region (Sang et al. 1997); 600 bp or 1200 bp of the chloroplast *rbcL* gene (Levin et al. 2003; Kress and Erickson 2007); chloroplast *ndhF* gene (Olmstead and Sweere 1994); intron region of a chloroplast tRNA gene (trnL) (Taberlet et al. 1991); and the external transcribed spacer (ETS) of the 18S–26S nuclear ribosomal DNA (Wright et al. 2001). The primers used for each region were those given in the publications cited. Any new-to-New Zealand plants were identified using a combination of morphological keys and genetic methods (James et al. 2014). Edited DNA sequences were compared against sequences from the GenBank database (Clark et al. 2016) using a BLAST search (Altschul et al. 1997).

Determining regulated taxa

To determine if taxa identified in our soil samples could be regarded by NPPOs as a biosecurity risk, we searched the online Pest Register maintained by MPI

(https://pierpestregister.mpi.govt.nz/). This resister includes information on pests and pathogens that can affect plant, animal or human health in New Zealand, including unwanted, notifiable, regulated and non-regulated organisms. Anything not listed on the register returns a 'no-record' response.

Data analysis

Relationships were analysed between various predictor variables (sample weight, freight type, sample origin, sample age, departure season) and response variables (counts of bacteria, fungi and *Pseudomonas*, and both counts and prevalence of nematodes, PPN and seeds). Prevalence (%) was determined as the percentage of samples containing each organism. Three different statistical programs were used, depending on the characteristics of data being analysed, with SAS used for negative binomial generalised linear models (GLMs) (SAS Institute Inc. 2011), R for zero-inflated count models (R Core Team 2019), and Minitab for binomial GLMs (Minitab Inc. 2010). Analyses of macro-invertebrate counts and prevalence were not performed because of extremely low prevalence in samples, and the very similar and small numbers recovered per sample could not provide any meaningful results for interpretation.

Predictor variables

Sample weights were treated in two ways for separate independent analyses: by classifying them into five classes (0–20g, 21–100g, 101–300g, 301–600g and 601–5000 g) for analysis as a factor; and by $\log_e$-transformation for analysis as a covariate. This is because despite sample weight being a continuous predictor, it is possible that its effect is not necessarily continuous. The sample weight class variable allowed the detection of non-continuous sample weight effects (e.g., only the largest weight class has a significant effect on a response variable while other weight classes do not).

Freight type was analysed as a factor with three levels: shipping container, flat rack container, or used machinery. Sample origin was independently analysed using three sets of classifications: country of origin of the sea freight, based on the shipping manifest (six specific countries, hence six levels). EcoRegion as defined by Terrestrial Ecoregions of the World (Oceania, Australasia, Nearctic, Neotropic, Palearctic, or Indo-Malay; there was no sample from the Afrotropic region) (Olson et al. 2001), and UNRegion as defined by United Nations geoscheme region (Asia, The Americas, Europe, or Oceania; there were no samples from Africa or the Middle East).

Sample age was roughly estimated as the date sampled at the New Zealand port minus the date of departure from the last port of call (days) and treated as a numerical variable.

Departure seasons were broadly classified into 'autumn' for freight that departed during February–April and 'winter' for June departures and 'spring' for November departures, since all last ports of call were in the Southern Hemisphere.

Response variables

Counts of bacteria, fungi and *Pseudomonas* were analysed using a generalised linear model (GLM) assuming negative binomial distributions through a log link function. The negative binomial GLMs were chosen because counts showed over-dispersion, which was beyond the level modelled by Poisson GLMs. The model included freight type for comparison and the $\log_e$-transformed sample weight covariate to adjust for weight differences among samples. The effects of the remaining predictor variables were then evaluated by stepwise inclusion, and this process was repeated by replacing the $\log_e$-transformed sample weight covariate with sample weight class factor. Interactions between the predictors were not included in the models. In each stepwise inclusion process, a predictor variable was added only if the variable explained a statistically significant amount of deviance assessed by its Chi-square statistic – that is, only if the significance level of the deviance was smaller than or equal to 0.05.

Counts of nematodes (total nematodes and PPN) and seeds were analysed using zero-inflated count models, which were a conditional combination of two GLMs: a GLM assuming a binomial distribution through a logit link function; and on the conditional to the binomial GLM, a GLM assuming negative binomial distribution through a log link function. The zero-inflated count model analysis was required to account for a large proportion of zero counts, which were not explained by a negative binomial GLM alone. Negative binomial models were used because these counts showed over-dispersion when the counts were not zero. For each variable, the zero-inflated count model analysis proceeded in the same way as for the bacterial, fungal and *Pseudomonas* counts by examining freight type effects first, which were adjusted using the $\log_e$-transformed sample weight covariate, followed by stepwise inclusion of other factors/variables. Interactions between the predictors were not included in the models. Then, the whole process was repeated by replacing the sample weight covariate with the categorical weight variable.

Prevalence of nematodes and seeds (percentage of samples in which each taxon was found) were analysed, using a GLM assuming binomial distributions through a logit link function. The binomial GLMs were appropriate for modelling a binary event, such as presence/absence of taxa. For prevalence analysis, freight type effects (adjusted for sample weight differences) were examined first, followed by stepwise inclusion of other factors/variables. Analysis was repeated using the sample weight factor term. Interactions between the predictors were not included in the models.

Position of soil on shipping containers

A separate set of analyses was conducted on counts and prevalence of different organisms in relation to position of samples on shipping containers. Position had four levels: roof, outer rail, underside and fix points. Fix points included forklift pockets and corner castings because the numbers of samples from these positions were small and their descriptive statistics suggested no apparent differences in sample weight or

organism counts and prevalence. The effect of position was examined first using the $\log_e$-transformed sample weight covariate for weight difference adjustment. This was followed by testing the effects of other predictors in the same stepwise process as described previously, then, repeated again by replacing the sample weight covariate with the weight factor. Negative binomial GLMs were used for counts of bacteria, fungi and *Pseudomonas*, zero-inflated negative-binomial count models were for counts of nematodes and seeds, and binomial GLMs were for prevalence of nematodes and seeds. These models were chosen due to the same reasons as described previously.

Availability of data and material

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Open access

Results

Soil collection and composition

A total of 191 soil samples were obtained from 145 shipping containers (168 samples), seven flat rack containers (9 samples) and 11 items of used machinery (14 samples) (Suppl. material 2: table S1). Used machinery included vacuum tankers, all-terrain vehicles, an excavator, a caravan and forklift tines placed on a flat rack. We also inspected shipping containers that had been profiled prior to arrival (including from Asia and North America) but were found to be clean. Flat rack containers had significantly higher (p <0.001) mean soil weights (2161.4 ± 756.65 g) than shipping containers (422.0 ± 65.98 g) and used machinery (321.1 ± 189.98 g). Summaries of sample weights by freight type are presented in Fig. 1. For all analyses using $\log_e$-transformed sample weight covariate, the overall median weight of 152.7 g was used for estimating the mean and standard error of the analysed count/prevalence.

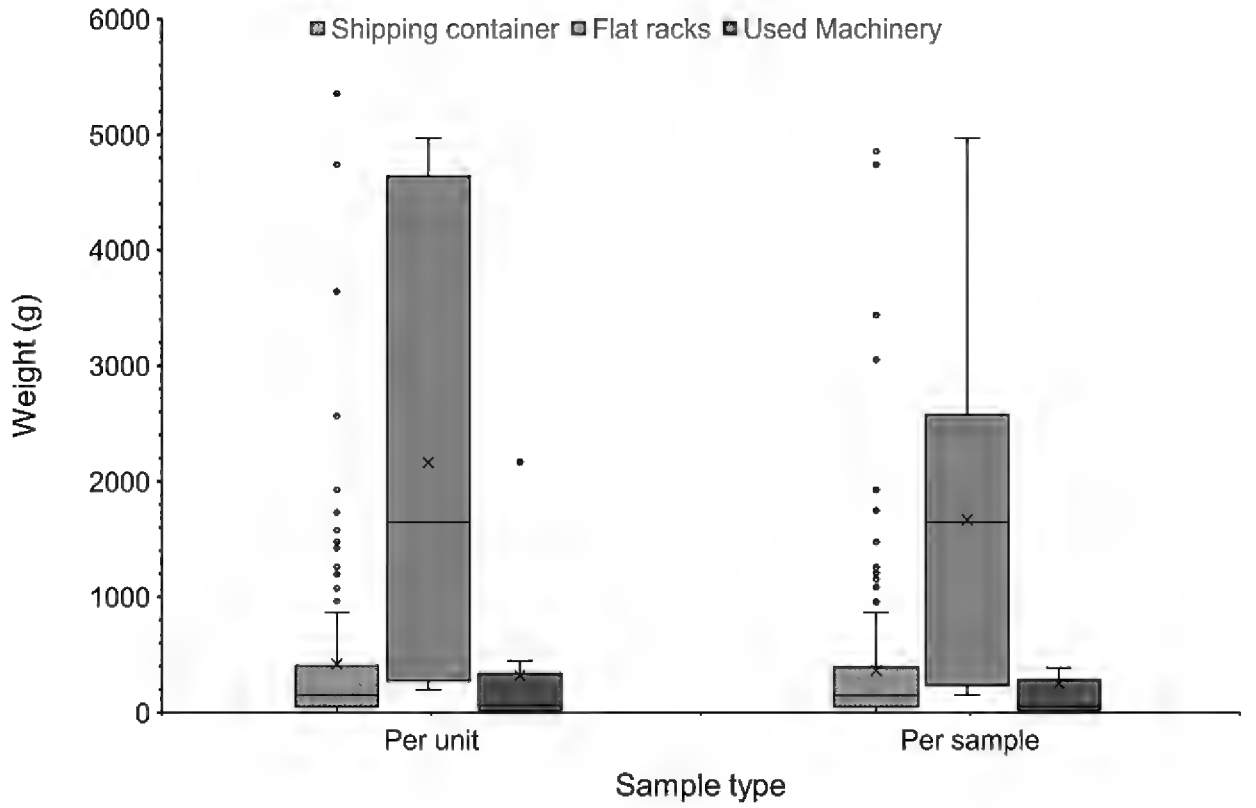

Figure 1. Boxplot indicating the amount of soil (g) (minimum, first quartile, median, mean, third quartile, and maximum) collected from shipping containers, used machinery and flat rack containers at New Zealand seaports per unit (individual freight or machinery item) and per sample, respectively.

Over 90% of shipping containers and flat racks (142/152) sampled originated from Australia (n = 36, 24%) and the Pacific Islands, with most island samples originating from Tonga (n = 59, 39%) and Vanuatu (n = 21, 14%) (Suppl. material 2: table S2). Used machinery originated predominantly from Australia (55%, n = 6), with the remaining five pieces from England, Wallis and Futuna Islands, Italy and USA.

Sixty-two percent of samples from shipping containers (104/168) were collected from under surfaces, mainly inner rails. The remaining samples were collected from corner castings (n = 8), forklift pockets (n = 12), outer rails (n = 36), roofs (n = 7), and the power unit bay of a refrigerated container (n = 1). Similarly, 78% of samples from flat rack containers (7/9) were from the underside, and the remaining two samples were from the container deck. On six occasions, soil was collected from multiple positions on shipping containers at the forklift pockets, underside (from both inner rails and container bottom) or outer rail. Samples collected from each container were kept separate for analysis. With used machinery, 64% of samples (9/14) were from vehicle undersides, with the remainder from the top of an excavator track (n = 1) and its associated stump-grinding head (n = 1), forklift tines (n = 2), and the upper cavities of a telehandler stabiliser points (n = 1).

All but one of 191 samples comprised a mixture of soil, compost, plant litter, plant debris, and non-organic matter components such as microfine to very coarse rocks (width range <1–20 mm), crushed coral and sand; the other sample consisted of bark pulp. Plant material in the samples comprised seeds, leaf material, plant fibres, woody fragments, and moss, either solely or mixed. The condition of grass leaves ranged from fresh to dry or senescing. Other organic and inorganic material in the samples included seashells, feathers, animal fibres, plastic, glass, metal, paint fragments and synthetic fibres.

Macro-invertebrates

Thirty eight of 191 samples (20%) contained live individuals, cadavers and/or body parts of insects, spiders, mites, a millipede and a springtail. Macroinvertebrates were found in soil collected from 27 shipping containers, three flat racks and four items of used machinery. Seven (4%) contained live specimens all recovered from shipping containers, and comprised a small range of taxa including a spider, mite, ants and parasitoid wasps (Suppl. material 2: table S3). No other taxa (e.g., annelids, molluscs) were found.

Culturable microorganisms

Bacteria, fungi and *Pseudomonas* were present in most samples (82–99%). Counts of bacteria and fungi were obtained from 184 of 191 samples. Due to both the spreading morphology of some bacteria and fungal overgrowth, accurate counts could not be obtained from some samples, and these were excluded. Bacteria were cultured from 181 of 184 samples (98%), with a mean (range) of 2.16×10^7 (0–7.24×10^8) CFU g^{-1} soil. *Pseudomonas* bacteria were isolated from 151 of 184 samples (82%) with a mean (range) of 8.83×10^4 (0–3.71×10^6) CFU g^{-1} of soil. Fungi were present in 182 of 184 samples (99%) with a mean (range) of 1.44×10^5 (0–4.09×10^6) CFU g^{-1} of soil.

Molecular identification of *Pseudomonas* bacteria

Just under half (70/152) of the soil isolates which produced colonies on PSA either yielded bacterial growth that contaminated the plates or yielded low numbers of colonies and were excluded from our investigation of the dominant *Pseudomonas* spp. A total of 119 isolates were used in the molecular identification of *Pseudomonas* species. Identical sequences that arose from morphological variants isolated from the same soil sample (7), and sequences outside of the Family Pseudomonadaceae (35), were removed from the analysis. The remaining 16S rRNA isolate gene sequences (77) were deposited with GenBank under the accession numbers listed in Suppl. material 2: table S4. Most *Pseudomonas* isolates (83%) came from shipping containers, with Tonga representing the most common country of origin (40%) (Suppl. material 2: table S4). Distance analysis of the 16S rRNA isolate sequences (Suppl. material 1: fig. S2) with reference species (Suppl. material 2: table S5) aided assignment of the isolates into described lineages and phylogroups (Suppl. material 2: table S5; Mulet et al. 2010; Peix et al. 2018; Passarelli-Araujo et al. 2022). All three *Pseudomonas* lineages (*P. aeruginosa*, *P. fluorescens* and *P. pertucinogena*) and 9/14 of the described phylogroups (Mulet et al. 2010; Peix et al. 2018; Passarelli-Araujo et al. 2022) occurred in the isolates (Suppl. material 2: table S4). Almost 64% (49/77) of isolates showed sequence similarity to the *P. aeruginosa* lineage, 35% (27/77) were most similar to the *P. fluorescens* lineage, and the remaining isolate (1/77) aligned with the *P. pertucinogena* lineage. Within the *P. aeruginosa* and *P. fluorescens* lineages, the most common phylogroups were *P. stutzeri* (80%, 39/49) and *P. putida* (70%,

19/27), respectively. No relationship was observed between country of origin and *Pseudomonas* species.

Nematodes and molecular identification

Most (187/191, 98%) samples were large enough to screen for nematodes after microbes had been extracted. Three samples contained only a small amount of soil which meant that that there was only enough for microbial extraction. The fourth sample was discarded due to a processing error during setup. Of 187 samples, 38% (72/187) contained nematodes. Total counts from the 72 samples ranged from 1 to 2130, with mean and median counts of 2.4 and 0.3 g^{-1} of soil, respectively. Bacterivores were the most diverse and numerous, followed by predators (Table 1). About half of samples with nematodes included genera classified as potential plant feeders such as *Aphelenchoides* (28), *Aphelenchus* (4) and *Ditylenchus* (14) (Table 1). Tylenchidae (10), which contains species associated with plant damage (Siddiqi 2000), were also present (Table 1). Sequencing results indicated nematode species included *Helicotylenchus dihystera* (Cobb) Sher); *Ditylenchus dipsaci* (Kühn) Filipjev (stem nematode); *Aphelenchoides besseyi* Christie (rice white tip nematode) or *A. fujianensis* Zhuo, Cui, Ye, Luo, Wang, Hu & Liao; *A. bicaudatus* (Imamura) Filipjev & Schuurmans Stekhoven; *A. fujianensis*; *Aphelenchus avenae* Bastion, and *Neopsilenchus magnidens* (Table 2).

Table 1. Incidence and number of live nematodes extracted from 72 soil samples removed from sea freight arriving at Auckland and Lyttelton sea ports. A total of 187 samples were used for nematode extraction.

Nematode taxa	Incidence in samples	Mean nos/ sample	Range
Bacterivores			
Cephalobidae	29	75	1–1160
Monohysteridae	2	16	14 & 18
Panagrolaimidae	48	143	1–1890
Plectidae	23	40	1–251
Prismatalaimus	1	16	–
Rhabditidae [a]	17	11	1–53
Rhabdolaimidae	2	9	1 & 16
Fungivores			
Aphelenchoides [a]	28	44	1–420
Aphelenchus [a]	3	8	2–13
Predators			
Diplogasteridae	1	10	–
Monochidae	1	8	–
Tobrilidae	2	17	2 & 32
Tripylidae [c]	3	32	2–84
Omnivores	20	29	1–201
Plant feeders			
Helicotylenchus [a]	2	1.5	1–2
Ditylenchus [ab]	14	10	1–30
Tylenchidae [ad]	10	59	1–500

[a] Taxa contains species regulated by New Zealand Ministry for Primary Industry (01 June 2023).
[b] Includes five samples in which a DNA match could not distinguish between *Anguina*, *Ditylenchus* or *Subanguina*.
[c] Separating Tobrilidae and Tripylidae morphologically is difficult.
[d] Excludes *Ditylenchus* and *Helicotylenchus*.

Table 2. Nematode species that were identified by molecular identification. Nematodes recovered from soil removed from sea freight arriving at Auckland and Lyttelton sea ports. Sequences that had 98% or better alignment to those listed on the BLAST website were considered to be a good match.

Possible plant feeders	Number of samples	Number sequenced	Results*
Helicotylenchus	2	3	3 *H. dihystera* (99%)
Ditylenchus	14	13	2 *D. dipsaci* (99%)
			2 *D. dipsaci* (96–97%)
			4 *Ditylenchus* sp. (<98%)
			5 *Anguina* or *Ditylenchus* or *Subanguina* [b](<97%)
Aphelenchoides	28	26	5 *A. besseyi* [a] / *A. fujianensis* (99%) [b]
			1 *A. bicaudatus* (99%) [a]
			1 *A. bicaudatus* (96%)
			2 *A. fujianensis* (99%)
			1 *A. saprophilus* [a] (96%)
			16 *Aphelenchoides sp.* (<96%)
Aphelenchus	3	4	4 *Aphelenchus avenae* (99+%)
Tylenchidae	10	7	1 *Neopsilenchus magnidens* (99%)
			6 *Filenchus* sp. (<98%)

* n refers to number matching the nematode species and % alignment;

[a] Regulated by New Zealand Ministry for Primary Industry (01 June 2023);

[b] Blast match unable to distinguish between these genera for these samples;

Note: Tylenchidae "Associates of algae, mosses, lichens and plant roots, but generally not root parasites of any significance" (Siddiqi 2000). *Ditylenchus* "Fungal feeders and parasites of higher plants, several species, including the type species, are capable of attacking aerial parts." (Siddiqi 2000).

Seeds and molecular identification

Seeds were present in 28% of samples (52/187), with a mean of 0.03 seeds g^{-1} soil across all samples, and a median and maximum of 0.00 and 1.7 seeds g^{-1} soil, respectively. Samples with seeds had a mean of 0.13 seeds g^{-1} soil with a median of 0.02 g^{-1} soil. Grass species predominated (61%, 589/974), followed by herbs (20%, 197/974) (Table 3), with 15 and 21 genera, respectively. DNA sequencing and morphological examination diagnosed 11 plant species (Suppl. material 2: table S6), of which seven were new records to New Zealand. These were *Ludwigia octovalvis* (Jacq.) Raven, *Hyptis pectinata* (L.) Poit., *Eriochloa procera* (Retz.) C. E. Hubb., *Fimbristylis cymosa* R. Br. var. *cymosa*, *Tribolium obliterum* (Hemsl.) Renvoize, *Euphorbia prostrata* Aiton, and *Cyperus iria* L.. All sequences that could be taxonomically assigned were deposited in GenBank (Suppl. material 2: table S6).

Comparing types of sea freight

There were no significant differences in bacteria counts in soil collected from shipping containers, flat rack containers and used machinery (Table 4). Mean counts of both fungi and *Pseudomonas* spp. were significantly higher in samples from shipping containers and used machinery than from flat rack containers (fungi, $p < 0.001$, Chi-square = 15.3, df = 2; *Pseudomonas*, $p < 0.001$, Chi-square = 37.5, df = 2). Counts of *Pseudomonas* spp. were significantly higher in used machinery than in shipping containers ($p < 0.001$).

Table 3. Plant taxa represented, numbers and viability of seeds recovered from soil removed from sea freight arriving at Auckland and Lyttelton sea ports.

Plant type	Total seeds	% viable seeds	*Genus*
Grass	589	70.6	*Axonopus*, *Brachiaria*[a], *Chloris*[a], *Cortaderia*[a], *Digitaria*[a], *Echinochloa*[a], *Eleusine*, *Eragrostis*[a], *Eriochloa*, *Megathyrsus*, *Panicum*[a], *Paspalum*[a], *Tribolium*, *Triticum*, unidentified
Herb	197	92.9	*Chenopodium*, *Cyclospermum*, *Eclipta*, *Eleutheranthera*, *Erigeron*, *Euphorbia*, *Fallopia*, *Hyptis*, *Hypochaeris*, *Lepidium*, *Ludwigia*[a], *Mimosa*, *Monopsis*, *Oldenlandia*, *Phyllanthus*, *Plantago*, *Polygonum*, *Portulaca*, *Sonchus*[a], *Symphyotrichum*, unidentified
Rush	13	100	*Juncus*
Sedge	60	96.7	*Cyperus*, *Fimbristylis*, unidentified
Trees	2	100	*Casuarina*
Unidentified	112	90.0	
Vine	1	100	*Macroptilium*

[a] Contains plant taxa regulated by New Zealand Ministry for Primary Industry (01 June 2023).

Nematode counts were significantly higher in samples from used machinery and shipping containers, than flat rack containers ($p < 0.001$, $Z = 4.9$ and 4.0, respectively), with no significant difference between shipping containers and used machinery (Table 4). Counts of PPN from used machinery were significantly higher than from both shipping containers and flat rack containers ($p = 0.004$, $Z = 2.91$, and $p = 0.003$, $Z = 2.94$, respectively). There was no difference in seed counts between freight types (Table 4).

Nematodes were significantly more prevalent in soil from used machinery than shipping containers ($p = 0.018$, $Z = 2.36$) and flat rack containers ($p = 0.028$, $Z = 2.20$). PPN were not significantly different. Seed prevalence was also significantly higher in samples from used machinery than from shipping containers ($p = 0.029$, $Z = 2.18$) (Table 4).

Culturable microorganisms

For bacteria, sample age had the greatest effect on bacteria counts ($p = 0.001$, Chi-square = 16.17, df = 1), with no additional effects of sample weight being found after the sample age effect was accounted for (Table 5). The predicted response was that mean bacterial counts declined by 25% per 1 day increase in sample age.

For fungi, weight class had the most dominant significant effect on counts ($p = 0.001$, Chi-square = 25.47, df = 4) (Table 5), with samples in three middle weight classes (21–100, 101–300 and 301 – 600 g) having larger fungal counts (1.62 ± 0.37, 1.61 ± 0.36 and $2.80 \pm 0.91 \times 10^5$, respectively) compared to samples in the other two classes: 0 – 20 g and 601 – 5000 g (0.63 ± 0.23 and $0.34 \pm 0.10 \times 10^5$, respectively). After weight class effects were accounted for, no other factor/variable had a significant effect on fungi counts.

For *Pseudomonas*, sample departure season has the most significant effect on counts ($p < 0.001$, Chi-square = 21.38, df = 2), with samples in autumn having smaller mean (±SEM) counts ($0.38 \pm 0.08 \times 10^5$) than samples in spring and winter (4.40 ± 3.31 and $1.26 \pm 0.36 \times 10^5$, respectively). After accounting for sample departure season effects, no other factor/variable was significant.

Table 4. Mean number (± SEM) and % prevalence (±SE %[1]) of bacteria, fungi, *Pseudomonas* (CFU g^{-1}), total and plant parasitic (PPN) nematodes, and plant seeds (g^{-1} of soil contaminant) in soil removed from different types of sea freight arriving at Auckland and Lyttelton sea ports. *P* and Chi square values were derived from either negative binomial GLM analysis or zero-inflated count model depending on the characteristics of data being analysed.

Organism taxa	Shipping container (168)	Flat rack (9)	Used machinery (14)	Significance
Bacteria	$1.95 \times 10^7 \pm 2.13 \times 10^6$ CFU^a	$1.19 \times 10^7 \pm 5.42 \times 10^6$ CFU^a	$2.74 \times 10^7 \pm 1.02 \times 10^7$ CFU^a	NS
	99.4%	100%	100%	NS
Fungi	$1.39 \times 10^5 \pm 1.84 \times 10^4$ CFU^a	$1.50 \times 10^4 \pm 9.09 \times 10^3$ CFU^b	$3.40 \times 10^5 \pm 1.62 \times 10^5$ CFU^a	$p < 0.001$
	98.8%	100%	100%	
Pseudomonas spp.	$5.35 \times 10^4 \pm 9.47 \times 10^3$ CFU^b	$1.31 \times 10^3 \pm 1.02 \times 10^3$ CFU^c	$7.84 \times 10^5 \pm 4.89 \times 10^5$ CFU^a	$p < 0.001$
	82.0%	77.8%	85.7%	
Total nematodes	0.48 ± 0.15^a	0.001 ± 0.001^b	6.96 ± 6.04^a	$p < 0.001$
	37 ± 3.7%^{b}	22 ± 14.6%^{b}	71 ± 12.6%^{a}	$p < 0.028$
PPN	0.02 ± 0.01^b	0.001 ± 0.001^b	0.65 ± 0.49^a	$p < 0.001$
	18 ± 3.0%^{a}	16 ± 12.8%^{a}	40 ± 13.6%^{a}	NS
Seeds	0.09 ± 0.02^a	0.03 ± 0.03^a	0.09 ± 0.06^a	NS
	25 ± 3.3%^{b}	36 ± 17.0%^{a,b}	54 ± 13.8%^{a}	p < 0.029

[1] The SE around the percentage mean *p* is calculated using the equation = $100^* \sqrt{\frac{\frac{p}{100}^*(1-p/100)}{n-1}}$, where n refers to number of samples. Values with the same letters not significantly different ($p > 0.05$).

Nematodes and seeds

The most dominant effect for nematode counts was weight class ($p < 0.001$, Chi-square = 33.0, df = 4) with samples in the 21–100g class having larger counts (2.73 ± 1.65 g^{-1} soil) than samples in other classes (e.g., 0–20g (0.24 ± 0.18), 101–300g (0.28 ± 0.10), 301–600g (0.22 ± 0.12) and 601–5000g (0.18 ± 0.13), respectively).

For PPN, $\log_e$-transformed weight covariate had the greatest effect on counts ($p = 0.012$, Chi-square = 8.8, df = 1), with the mean number of PPN decreasing by 41% for every 1 unit increase in $\log_e$ weight. The predicted response for mean PPN counts was that if 152.7 g soil (overall median soil weight) contained 100 PPN g^{-1} soil, 415.0 g soil would contain 59 PPN g^{-1} soil.

For prevalence of total nematodes, UNRegion had the most significant effect ($p = 0.002$, Chi-square = 14.80, df = 3, binomial GLM) compared to the other significant variables with the highest prevalence in soil originating from Europe (100%, n = 3), followed by Oceania (39 ± 3.7%, n = 161), whereas there were no nematodes in soil from Asia and the Americas (0%, n = 9). For PPN prevalence, no variable was found to be significant.

For seed counts and seed prevalence, no factors/variables had any significant effects (Table 5).

Position of soil on shipping containers

Overall, there were no significant differences in bacterial, fungal and *Pseudomonas* counts in soil collected from sea container roofs, outer rails, undersides and fix points (Table 6). However, nematode counts were absent in soil from roofs, with this absence

Table 5. Significance levels (*p*-values) of the effects of sample weight (class factor or $\log_e$-transformed covariate), sample origin (country, EcoRegion or UNRegion), sample age and sample departure season on counts of bacteria, fungi, *Pseudomonas*, and counts and % prevalence of total and plant parasitic (PPN) nematodes, and seeds, associated with soil removed from sea freight arriving at Auckland and Lyttelton sea ports. The most significant influencing variable indicated in bold.

Organism taxa	Sample weight		Sample origin			Sample age	Departure season
	Class factor	Covariate	Country	EcoRegion	UNRegion		
Bacterial count	< 0.001	< 0.001	0.793	0.203	0.219	**< 0.001**	0.287
Fungal count	**< 0.001**	0.862	0.040	0.045	0.195	0.118	0.235
Pseudomonas spp. count	0.003	0.689	< 0.001	0.007	0.013	0.014	**< 0.001**
Total nematodes count	**< 0.001**	< 0.001	0.019	< 0.001	0.014	0.201	0.002
Prevalence	0.221	0.022	0.101	0.009	**0.002**	0.064	0.591
PPN count	0.017	**0.012**	0.190	0.019	0.407	0.183	0.406
Prevalence	0.384	0.103	0.190	0.054	0.324	0.115	0.944
Seed count	0.647	0.150	0.429	0.221	0.111	0.527	0.165
Prevalence	0.450	0.115	0.940	0.760	0.842	0.737	0.774

Table 6. Mean number (± SEM) and % prevalence (±SE %[1]) of bacteria, fungi, *Pseudomonas* (CFU/g soil), total and plant parasitic (PPN) nematodes, and seeds (/g soil), by position on shipping containers. Comparisons were made in either negative binomial GLM analysis or zero-inflated count model analysis for numbers, depending on the characteristics of data being analysed. For prevalence, comparisons were by binomial GLM analysis. Prevalence of bacteria, fungi and *Pseudomonas* spp. were generally very similar, therefore not statistically compared.

Organism taxa	Roof (7)	Outer rail (36)	Underside (104)	Fix points (20)
Bacteria	$1.3 \times 10^7 \pm 8.44 \times 10^6$ CFU[a]	$1.7 \times 10^7 \pm 4.22 \times 10^6$ CFU[a]	$2.2 \times 10^7 \pm 3.13 \times 10^6$ CFU[a]	$1.8 \times 10^7 \pm 5.87 \times 10^6$ CFU[a]
	100%	97.2%	100%	100%
Fungi	$1.1 \times 10^5 \pm 8.12 \times 10^4$ CFU[a]	$1.2 \times 10^5 \pm 3.62 \times 10^4$ CFU[a]	$1.7 \times 10^5 \pm 2.79 \times 10^4$ CFU[a]	$7.9 \times 10^4 \pm 3.13 \times 10^4$ CFU[a]
	85.7%	100%	99.0%	100%
Pseudomonas	$2.9 \times 10^4 \pm 2.88 \times 10^4$ CFU[a]	$3.7 \times 10^4 \pm 1.69 \times 10^4$ CFU[a]	$6.7 \times 10^4 \pm 1.55 \times 10^4$ CFU[a]	$3.9 \times 10^4 \pm 1.99 \times 10^4$ CFU[a]
	85.7%	83.3%	84.6%	73.7%
Total nematodes	0.0[c]	0.5 ± 0.26[a,b]	0.2 ± 0.06[b]	1.5 ± 0.86[a]
	0.0%[b]	32 ± 8.6%[a]	39 ± 4.9%[a]	42 ± 11.2%[a]
PPN	0.0[c]	0.13 ± 0.07[a]	0.02 ± 0.005[b]	0.10 ± 0.07[a,b]
	0.0%[a]	19 ± 7.6%[a]	25 ± 5.2%[a]	22 ± 9.7%[a]
Seeds	0.01 ± 0.01[a]	0.03 ± 0.01[a]	0.03 ± 0.01[a]	0.03 ± 0.02[a]
	38 ± 21.9%[a]	29 ± 8.2%[a]	22 ± 4.1%[a]	34 ± 10.7%[a]

[1] The SE around the percentage mean p calculated using the equation = $100^* \sqrt{\frac{\frac{p}{100} * (1 - p/100)}{n-1}}$ Letter n in the equation refers to number of samples. Values with the same letters are not significantly different ($p > 0.05$).

being significant against other positions, while the counts from fix points (1.5 ± 0.86 g^{-1} soil) were significantly higher than those from sea container undersides ($p < 0.001$, Chi-square = 18.8, df = 2) (Table 6).

Although numbers of PPN g^{-1} soil were low (< 0.13 g^{-1}soil), significant differences in counts were found between positions, with PPN counts significantly higher ($p < 0.001$, Chi-square = 16.6, df = 2) in soil from outer rails, undersides and fix points compared to roof soil, where no nematodes were recovered. Significant differences were also found between the underside and outer rails ($p = 0.012$, $Z = 2.53$). The number of seeds g^{-1} of soil did not significantly differ between the four positions (Table 6).

Total nematode prevalence was highest in container fix points (42 ± 11.2%), followed by undersides (39 ± 4.9%), and outer rails (32 ± 8.6%), with no nematodes found in the roof soil. The absence in the roof soil was significant ($p < 0.001$, Chi-square = 22.5, df = 2) against the other positions. The prevalence of PPN was relatively low, with no significant differences amongst shipping container positions. Seed prevalence did not significantly differ between the four positions, either (Table 6).

Shipping containers alone

The sea container analysis showed similar relationships to the full dataset and detailed in Suppl. material 3.

Discussion

Trade and commerce have been identified as key elements contributing to the spread of exotic species on a global scale. Not only does the spread of exotic species through these networks represent significant environmental, economic and social costs to natural and agricultural environments if IAS (a subset of exotic species) were to establish, a loss of biodiversity is also an expected consequence of IAS establishment. For islands, the implications can be significant, as they have high levels of endemism and IAS establishment can lead to extinction of species as well as biodiversity declines (Pyšek et al. 2020; Dawson et al. 2022).

The global movement of sea freight has an important role in the spread of IAS. In this study, it was found that soil on sea freight supported viable taxa either found in the soil (e.g., microorganisms, nematodes) or associated with the soil (e.g., arthropods). Soil was present on most types of sea freight, irrespective of origin, with all soil likely to vector microbes including plant pathogenic ones. About a third of soil contaminants contained live nematodes, some of which are plant feeders. Some of the taxa recovered are regulated organisms or found to be new interception records (e.g., *Pseudomonas*, nematodes, plants) under New Zealand biosecurity legislation. Importantly, this reinforces the importance of phytosanitary measures either pre- or at the border, to remove risk organisms and minimise the flow and potential for establishment of IAS. Seventy-three percent of soil was collected from the undersides of containers and flat racks, which was consistent with a previous shipping container survey that found most soil on container undersides (Gadgil et al. 2000). Similarly, 70% of soil in our study was obtained from shipping containers arriving from Pacific Islands. Gadgil et al. (2000) also found high rates of soil contamination on Pacific Island shipping containers. Although we also inspected containers from other regions (e.g., Asia and North America), superior hygiene standards in those regions meant the incidence of soil contamination was very low (and so not sampled). Flat rack containers had the highest soil contamination compared to shipping containers and used machinery, possibly because their design and role in carrying a wide range of freight in an open environment meant

a higher probability of being contaminated. However, despite a higher soil loading, it was found that the prevalence of fungi, *Pseudomonas* spp., nematodes and seeds were significantly lower than the other freight types. Counts of fungi, *Pseudomonas* spp., and nematodes were also significantly lower in flat rack containers compared to the two other freight types.

In terms of the variables analysed, the result was found to vary with taxa. Sample age was shown to have the most impact on survival of bacteria, while sample weight was the most important parameter for fungal counts, with a bell curve distribution whereby counts were significantly lower at both 0–20 g and >600 g weight classes, compared to weights >20–600 g. Why this trend occurred is not clear but may be an artefact of soil type or environmental exposure, or the interaction of both variables. For *Pseudomonas* counts, samples collected in spring and winter were significantly higher than autumn. For nematode counts, weight class was significant, while nematode prevalence was influenced by the region from which the soil originated, highest in soil from Europe. However, the small sample size from Europe (n = 3) meant that the result should be treated with caution. For PPN, $\log_e$-transformed weight covariate had the greatest effect on counts.

When compared with the footwear study (McNeill et al. 2011), there were marked differences in the weight of soil collected and number and incidence of taxa. Perhaps not surprisingly, mean sample weight from sea freight was 417.3 g compared to 3.2 g for footwear (Table 7). While counts of culturable bacteria and fungi were comparable with those found on contaminated footwear, the incidence of nematodes, seeds and arthropods was higher in soil taken from footwear. Similarly, the number of nematodes and seeds per gram of soil was 45 × and 83 × higher in soil from footwear in comparison to sea freight (Table 7). Soil weight was the significant variable for fungi counts for both sea freight and shipping containers. However, in the footwear study, season was the most significant variable with higher fungi counts for samples collected in summer than in autumn.

A previous, controlled experiment that examined storage time and environmental exposure on survival of soil biota associated with shipping containers (McNeill et al. 2017), concluded that in general, the survival of soil biota is reduced by both duration of transport and exposure to sunlight, temperature and moisture fluctuations. Greater exposure of soil to abiotic parameters may explain why flat racks, despite supporting greater volumes of soil, had significantly lower counts and incidence of live taxa compared to shipping containers and used machinery. However, counts of fungi and bacteria can also be high from soil in exposed situations, probably due to ongoing colonisation by airborne propagules (McNeill et al. 2017). The sea freight results for nematodes were generally consistent with those of McNeill et al. (2017), because the percentage of soil found with nematodes was highest in the forklift points, the underside and outer rail, whereas prevalence was zero in roof soil. The prevalence of PPN was highest in soil collected from the underside of containers with no significant differences amongst the remaining shipping container positions. However, unlike McNeill et al. (2017) - but as in the footwear study (McNeill et al. 2011) - bacteria counts significantly declined with increasing soil age. Possibly the increase in fungal counts with time observed by

Table 7. Summary of organisms found associated with soil on sea freight in comparison with previous results from footwear.

	Sea freight	Footwear[a]
Number of samples	191	155
Sample weight (g)		
Mean per sample	417.3	3.2
Median per sample	152.7	1.0
Bacteria		
Bacteria incidence	0.99	1.00
Bacteria (CFU/ gram of soil)	2.1×10^7	2.9×10^7
Fungi		
Fungi incidence	0.99	0.98
Fungi (CFU/ gram of soil)	1.4×10^5	5.6×10^5
Nematodes		
Nematode incidence	0.38	0.65
Mean nematodes/ gram of soil	0.9	41
Seeds		
Seed incidence	0.28	0.52
Mean seeds/ gram of soil	0.03	2.5
Proportion grass	0.67	0.44
Proportion herb	0.23	0.13
Proportion trees and shrubs	0.02	0.39
Arthropods		
Incidence of arthropods or arthropod-parts	0.20	0.38
Proportion of arthropods alive	0.18	0.03

[a] McNeill MR, Phillips CB, Young S, Shah F, Aalders L, Bell N, Gerard E, Littlejohn R (2011) Transportation of nonindigenous species via soil on international aircraft passengers' footwear. Biological Invasions 13: 2799–2815. https://doi.org/10.1007/s10530-011-9964-3.

McNeill et al. (2017) occurred because their experiment was conducted entirely on land where airborne fungal spores may be more abundant than at sea where the sea freight samples in the present study spent some of their time. This study found no significant differences in counts or prevalence of seeds, bacteria, fungi and *Pseudomonas* spp. amongst samples sampled from four different locations on sea containers.

Nineteen different nematode families or species were found in sea freight soil, which was comparable to the results of the footwear study (McNeill et al. 2011), where six trophic groups containing 17 families or species were found. The types of nematodes detected were also similar with Rhabditidae, *Panagrolaimus*, Plectidae, *Aphelenchoides*, *Aphelenchus*, *Ditylenchus* and Omnivores being common to both studies. As noted previously, nematode counts in soil from sea freight were significantly higher in soil between 21–100g, compared to weights below and above the range. By comparison, in footwear there was a significant increase with increasing soil weight (McNeill et al. 2011).

Various species of PPN were identified from the samples, some of which are regulated pests in New Zealand, thus showing that sea freight can act as a pathway for the movement for regulated species. *Aphelenchoides besseyi* (regulated) causes white tip disease in rice and 'summer crimp' in strawberries, and also feeds on other plants and fungi (Hunt 1993). *Aphelenchoides fujianensis* (regulated) occurred in soil on a shipping container from Vanuatu; little is known about this species, but it is probably

mycophagous (Zhuo et al. 2010). Another shipping container specimen from Vanuatu was probably *A. bicaudatus* (regulated), which feeds on cultivated mushrooms (Hunt 1993). *Aphelenchus avenae* has been previously recorded in New Zealand and is associated with mushrooms (Knight et al. 1997). *Neopsilenchus magnidens* (Thorne) Thorne & Malek, was collected off used machinery from Italy and is associated with alfalfa in the USA (Siddiqi 2000). *Helicotylenchus dihystera* (Cobb) Sher, found in soil from Tonga, and *Ditylenchus dipsaci* (Kühn) Filipjev (stem nematode), recovered from both a shipping container from Tonga and from a stump grinding excavator from Australia are already present in New Zealand (Knight et al. 1997).

Pseudomonas isolates, representing the most dominant colony types on PSA for each sea freight soil sample, covered the three *Pseudomonas* lineages and nine of the fourteen phylogroups (Mulet et al. 2010; Passarelli-Araujo et al. 2022; Peix et al. 2018). *Pseudomonas stutzeri* was the most dominant phylogroup (51%) followed by the *P. putida* phylogroup, (25%) (Suppl. materials 1, 2: fig. S2, table S4). Isolates were not identified to species level due to the complexity of *Pseudomonas* taxonomy with species identification requiring the sequencing of at least three house-keeping genes in addition to the 16S rRNA gene (Mulet et al. 2010). Furthermore, testament to the rapidly evolving field of *Pseudomonas* taxonomy is the proposal of several new genera within the Pseudomonadaceae family which include species formerly ascribed to the *Pseudomonas* genus (Hesse et al. 2018; Gomila et al. 2022; Lalucat et al. 2022). A general understanding of the genetic diversity within the *Pseudomonas* isolates was therefore appropriate for this study.

Twenty-five plant-pathogenic *Pseudomonas* spp. have been described (Höfte and De Vos 2007; Bull et al. 2010, 2012), with *P. syringae* van Hall, considered a bacterial plant pathogen with significant impact (Mansfield et al. 2012). Godfrey and Marshall (2002) previously showed that exotic soil attached to shipping containers was a potential source of new pseudomonad biodiversity into New Zealand. Neither *P. stutzeri* nor *P. putida* are known as plant pathogens (Bull et al. 2010, 2012). *Pseudomonas stutzeri* occupies diverse ecological niches and has been isolated as an opportunistic pathogen from humans (Lalucat et al. 2006). *Pseudomonas putida* is a metabolically versatile saprophytic soil bacterium and appears to lack genes for the proteins and enzymes that cause disease in plants (Nelson et al. 2002). Five isolates (AUS50i, AUS139b, AUS140a, ITA154c and UNK162a), that originated from Australia, Italy and the United Kingdom, grouped with the *P. fluorescens* phylogroup which is closest to the *P. syringae* phylogroup where the highly virulent plant pathogenic *P. syringae* pathovars reside (Suppl. material 1: fig. S2). The results of the sequence analysis demonstrated the high species diversity in the *Pseudomonas* sea freight isolates and the potential for soil on sea freight to transport high risk species.

The diversity of *Pseudomonas* spp. found in this study also aligns well with Godfrey and Marshall (2002), who found a wide distribution of soil isolates throughout the *Pseudomonas* genus from imported shipping containers. In contrast, an earlier study on shipping containers arriving at New Zealand ports showed much lower *Pseudomonas* spp. diversity (Marshall and Varney 2000). In both the Marshall and Varney (2000) and Godfrey and Marshall (2002) studies, *P. putida* was the dominant species.

Twenty eight percent of samples contained seeds including the genera *Brachiaria*, *Cortaderia*, *Digitaria*, *Eragrostis* (grasses) and *Sonchus* (herb), which include biosecurity risk species regulated in New Zealand. Grass and herb genera (n = 16 and 21, respectively) were the dominant plant types, which was a similar diversity to the footwear study (McNeill et al. 2011). However, there was little commonality in the genera found with only three grass (*Eragrostis*, *Paspalum*, *Triticum*) and one herb (*Polygonum*) genus occurring in both studies. This dissimilarity may have arisen from geographic differences in the sample sources: sea freight was mainly from the Pacific Islands and Australia, whereas footwear was mainly from the UK, Australia, Central Europe and North America. The difference may also relate to the mobility of humans and the potential for footwear to be contaminated from more diverse environments, compared to that of sea freight which is mainly associated with road and rail transport networks.

Only 20% of the samples contained live and recognizable dead arthropods, but they included taxa that are known to, or have potential to, impact native fauna (e.g., spiders, Eulophid wasps), are invasive (e.g., ants) and potential impacts on animal health (e.g., flies). The wasp nest containing the cadaver of eumenid wasp *Delta esuriens* was of interest. The wasp is introduced to the Pacific, and while reported in the Cook Islands and Samoa, was not known from Tonga from which the container originated. *Delta esuriens* has not been reported in New Zealand.

Whether these results allow for development of recommendations for prioritising one pathway over another, to increase the efficiency and efficacy of biosecurity interventions is open to debate. Comparison of sea freight compared to footwear indicate that both pathways vector biosecurity risk organisms, although the level of risk did vary, with the incidence, counts and diversity in soil on external surfaces of shipping containers less than soil transported in more protected environments (e.g., on footwear in luggage). Prioritising effort must also be viewed in relation to other pathways along which exotic species can move (Hulme et al. 2008). The conservative approach would deem both pathways important and therefore require biosecurity intervention. This is particularly so because interventions (e.g., cleaning), address a range of taxa collectively posing a potential biosecurity risk. In addition, pathways for biological invasions are dynamic and priorities may shift depending on new information, such as new pests or diseases (Barnes et al. 2018), or secondary invasions that provides new pathways for exotic species to disperse (Bertelsmeier and Keller 2018).

Over 2011 and 2012, the years in which the study was undertaken, approximately 456,680 empty and 750,500 full containers arrived at New Zealand seaports (MPI, unpublished data). Of these, the majority of sea containers were from Australia (c. 33%), followed by China (13%), Singapore (9.4%) and Malaysia (7%). The Pacific Islands were the origin for 6.3% of the sea container freight. Over the same time frame, c. 20,000 used vehicles were imported into the country. External contamination of shipping containers has been found to vary from 1 (M. R. McNeill, unpublished data) to 10% (Anon, 2016), and 1–50% for used vehicles (Hustedt 2010). This study therefore sampled a fraction of the shipping containers and used machinery arriving in New Zealand each year that carried some form of contamina-

tion. Nevertheless, we were able to detect numerous diverse exotic organisms, some of which were regulated pests.

In 2011, the volume of TEUs (20-foot equivalent units) moved globally by maritime shipping was 153 million, but grew by 4.6% in 2013, taking total volumes to 160 million TEUs (UNCTAD 2014, page 17). By 2018, containerised trade reached 196 million TEUs (Clarksons Research 2019), and while the Covid-19 pandemic and trade tensions have impacted maritime trade (UNCTAD 2020), Clarksons Research predicted trade volumes would reach 206.8 million TEUs in 2021 (Chambers 2021). Even if only 1% of shipping containers and sea freight were contaminated with soil, our results suggest they must facilitate significant exchange of exotic species across and within countries. Our results therefore support Dawson et al. (2022), who found that shipping containers were the primary introduction pathway for IAS to United Kingdom Overseas Territories. Managing the risk posed by sea freight is critical if a country's environment, biodiversity and economy is to be protected.

Acknowledgements

The authors thank the staff from the Ministry for Primary Industry (MPI), Ports of Auckland and Lyttelton Port of Christchurch for their support in this study, Cor Vink (AgResearch) for identification of spiders, Claire Dowsett (AgResearch) for rearing of plant material, and Trevor Crosby and Darren Ward (Landcare) for identification of live Diptera and Hymenoptera, respectively. Special thanks to MPI staff, especially Bridget Roberts for assistance in identifying freight to sample and her support for this project. AgResearch internal referees Chris Buddenhagen and Nicholas Davis provided thoughtful advice on an earlier draft of this manuscript. We also thank anonymous reviewers for valuable comments and suggestions on the submitted journal draft. This research was funded by the Ministry of Science and Innovation through the Better Border Biosecurity programme (www.b3nz.org), with the support of the Ministry for Primary Industries (MPI) and Department of Conservation (DOC).

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Supplementary material 1

Sampling points on sea container and visualisation of the genetic diversity between the 16S rRNA genes

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Data type: pdf

Explanation note: **fig. S1.** Sampling points on sea container indicating roof, outer rail, underside (inner rails or bottom of container), fork lift pockets and corner castings. Examples of contamination recorded during the study are shown. **fig. S2.** Visualisation of the genetic diversity between the 16S rRNA genes of 77 sea freight soil isolates and 21 *Pseudomonas* reference strains. The isolates from this study are named using the GenBank accession numbers (KX670872-KX670948) followed by a three-letter acronym denoting the country that the shipping container originated from: AUS Australia, CHR Christmas Island, FIJ Fiji, ITA Italy, MAL Malaysia, SAM American Samoa, TAI Thailand, TGA Tonga, UNK United Kingdom, USA United States of America, VAN Vanuatu and WFI Wallis & Futuna Islands. Phylogroups fall in the following *Pseudomonas* lineages: *P. aeruginosa* (*P. acaligenes*, *P. aeruginosa*, *P. oryzihabitans*, *P. oleovorans*, *P. stutzeri*), *P. fluorescens* (*P. anguilliseptica*, *P. fluorescens*, *P. putida*, *P. syringae*) and *P. pertucinogena* (*P. pertucinogena*). Using MEGA11, distance was calculated by the Jukes-Cantor model and visualised using a neighbour-joining tree. The robustness of the nodes was assessed with 1000 bootstrap replicates and values showing ≥50% support are shown.

Link: https://doi.org/10.3897/neobiota.88.98440.suppl1

Supplementary material 2

Soil contamination data, Insect taxa, Pseudomonas isolates and reference species, and plant taxa identified by molecular analysis

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Data type: pdf

Explanation note: **table S1.** Details on amount of soil (g) collected from shipping containers, flat rack containers and used machinery at Auckland and Christchurch sea ports per unit (individual freight or machinery item) and per sample. **table S2.** Number and weight of soil removed from sea container and flat rack containers sampled at Auckland and Lyttelton sea ports. **table S3.** Live insect taxa recovered directly from soil removed from shipping containers arriving at Auckland and Lyttelton sea ports. **table S4.** *Pseudomonas* isolates from soil removed from sea freight arriving into Auckland and Lyttelton sea ports. Assigned to previously described *Pseudomonas* phylogroups and lineages (Mulet et al. 2010; Peix et al. 2018; Passarelli-Araujo et al. 2022). **table S5.** Twenty-one reference species of the genus *Pseudomonas* used in the 16S rRNA gene nucleotide-based distance analysis. Isolated from soil recovered from sea containers arriving at Auckland and Lyttelton sea ports. **table S6.** Plant species identified by molecular analysis from soil collected from sea freight arriving at Auckland and Christchurch sea ports.

Link: https://doi.org/10.3897/neobiota.88.98440.suppl2

Supplementary material 3

Analysis of shipping container data only

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Data type: docx

Explanation note: Effects of soil weight, origin, source, sample age and season bacteria, fungi and *Pseudomonas*.

Link: https://doi.org/10.3897/neobiota.88.98440.suppl3